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MECHANISM OF THE EXCITATION-CONTRACTION UNCOUPLING OF FROG SKELETAL MUSCLE BY FORMAMIDE

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ABSTRACT

The contractility of guinea pig ileum and frog skeletal muscle is inhibited in solutions containing 0.4 to 2.5 *M* formamide (FMD). Contrary to mammalian visceral muscle, this blocking action is not reversed when frog muscles are transferred back to isotonic Ringer's after FMD treatment. Under these conditions the water content of the skeletal muscles is markedly increased and electronmicrographs show a swelling of the transverse tubules. These changes are not observed when frog muscles are transferred to ethylene glycol solutions that are isosmotic with the FMD containing Ringer's solution. In addition, over 50% of the contractility is recovered in these muscles. These observations provide direct evidence of the occurrence of an osmotic shock in frog muscles transferred from FMD solutions to isotonic Ringer's. It is concluded that the resulting alterations in the triad structure and function are responsible for the irreversibility of the FMD uncoupling action in these muscles.

INTRODUCTION

Formamide (FMD), added to isotonic saline at concentrations between 0.4 and 2.5 *M*, produces an immediate and completely reversible inhibition of the shortening of mammalian visceral muscle (Córdoba *et al.*, 1968) and blocks irreversibly with a slower time course the contractility of frog skeletal muscle (del Castillo and Escalona de Motta, 1978). The skeletal muscle fibers blocked by FMD retain their electrical and chemical excitability properties and are able to respond with fast local twitches to the electrophoretic injection of Ca^{2+} (Escalona de Motta and del Castillo, unpublished observations). In addition, caffeine still induces slow sustained contractures in these muscles (Escalona de Motta *et al.*, 1982). These observations suggest that the effects of FMD on frog skeletal muscle are exerted on the coupling between excitation and contraction. In this sense, FMD may be classified, together with glycerol and ethylene glycol, as an excitation-contraction (E-C) uncoupling agent.

However, the uncoupling action of glycerol and ethylene glycol does not occur until the muscles are suddenly transferred back to isotonic Ringer's, inducing an osmotic shock that disrupts the tubules of the T-system. (Eisenberg and Gage, 1967). With FMD, muscles lose their contractility while still immersed in the hypertonic

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Abbreviations: E-C, excitation-contraction; EG, ethylene glycol; FMD, formamide; SR, sarcoplasmic reticulum.

solution, indicating that an osmotic shock is not essential for the uncoupling action exerted by FMD (del Castillo and Escalona de Motta, 1978). The present work investigates the possible osmotic effects of FMD solutions on frog sartorius muscles, measuring changes in the water content of these muscles and examining the ultrastructure of the muscle fibers under various conditions.

Materials and Methods

Preparations. Sartorius muscles of small (2″) frogs (*Rana pipiens*) were dissected, with or without the sciatic nerve attached, and pinned to a layer of Sylgard (Dow Corning) at the bottom of a small Petri dish. Contractility was determined visually by observing the twitches induced by stimulating the muscle directly or via the attached motor nerve using a pair of platinum electrodes. All the applied stimuli were square pulses of 1 ms duration and supramaximal strength. In experiments where the tension developed was measured, the muscles were tied at both ends with silk threads and placed in a vertical bath containing Ringer's solution. The muscle, attached to the chamber by one end, was connected by the other end to a Grass FT 103 isometric transducer connected to a chart recorder.

Physiological solutions. The mammalian Krebs-Ringer's solution employed had the following ionic composition (m*M*): Na^+, 118; K^+, 4.6; Ca^{2+}, 2; Mg^{2+}, 0.9; Cl^-, 117; HCO_3^-, 17.6; SO_4^{2-}, 0.9; $H_2PO_4^-$, 0.9. A mixture of 98% 0_2 and 2% CO_2 was bubbled continuously through the solution, which had a pH of 7.3 after equilibration. Glucose (5 m*M*) was added to this solution. Direct measurement of osmolarity with a cryosmometer gave values ranging between 295 and 305 mOsm/liter.

The frog Ringer's solution used contained the following ionic concentrations (m*M*): Na^+, 117; K^+, 2.1; Ca^{2+}, 1.87, all as chloride salts. The pH of this solution was adjusted to 7.2 with 5 m*M* TES (N-tris hydroxymethyl methyl-2- aminoethanesulfonic acid) and NaOH. The osmolarity of this solution, determined with an Advanced Instruments osmometer, ranged from 242 to 250 mOsm/liter. FMD or ethylene glycol (EG) was added to these solutions in the concentrations further indicated.

Measurement of water content in muscle. The muscles were weighed at regular intervals before and after they were immersed in Ringer's solution to which different amounts of FMD had been added. Extreme care was taken in handling the muscles to ensure reproducibility of the results. After each series, the muscle was dried over a desiccant, until there was no further change in weight. The water content of each muscle was calculated by subtracting the dry weight from the original wet weight.

Ultrastructural experiments. Muscles which were in normal Ringer's saline were fixed in a solution containing 2% glutaraldehyde in 0.05 *M* cacodylate buffer (pH 7.4) with 4% sucrose for 24 hours at 4°C, and the solution was changed several times during the first hours.

Other muscles were transferred directly from a 2.0 *M* FMD solution in Ringer's to a fixative like the one above, but also containing 2.0 *M* FMD.

Finally, a third group of muscles was sequentially transferred from 1.0 *M* FMD to isosmotic ethylene glycol and then to the basic fixative solution to which 1.0 *M* of ethylene glycol had been added.

All specimens were treated subsequently with 1% osmium tetroxide in 0.05 *M* cacodylate buffer for 1 hour at 4°C, dehydrated in an ethanol series, and embedded in Araldite. Sections were cut with glass or diamond knives on an LKB Ultratome III, stained with ethanolic uranyl acetate and lead citrate, and examined in a Philips EM 400 and 200.

Results

Osmotic effects of high FMD concentrations. FMD is a highly permeant solute, as shown by the fact that the water content of ileal strips placed in 2 *M* FMD in Krebs-Ringer's solution show no appreciable change in weight after 20 min. In addition, as shown in Figure 1, no significant changes in water content could be detected when these ileal strips were transferred back to normal Krebs-Ringer's.

The movement of FMD across the membrane systems of skeletal muscle is slightly more restricted. Indeed, frog sartorius muscles exhibit a small (15%) decrease in total weight when immersed in 2 *M* FMD-Ringer's, and almost double their water content when transferred back to normal Ringer's (see Figure 2). This last observation suggests that FMD does not leave the skeletal muscle fibers as easily as it goes into them, thus favoring the occurrence of an osmotic shock similar to that brought about in skeletal muscles exposed to hypertonic glycerol (Gage and Eisenberg, 1969).

Ultrastructure of muscles equilibrated in hypertonic FMD. To determine whether the block of contraction which occurs while muscles are still immersed in the FMD solution could be correlated with ultrastructural changes caused by hypertonicity, two muscles blocked after 18 min in 2.0 *M* FMD were transferred to a fixing solution that also contained 2.0 *M* FMD. Post-fixation and further processing of these specimens was then done as described in Materials and Methods. Figures 3 and 4 show that the myofibrils and associated sarcoplasmic reticulum (SR), as well as the membranes of the tubular system (T-system), of these muscles are normal in appearance and do not present any obvious morphological features that may be associated with a loss of contractility.

Structural changes in FMD-blocked muscles transferred to normal Ringer's. Two muscles blocked after 18 min in 2 *M* FMD-Ringer's were transferred to isotonic Ringer's and washed extensively for 30 min before fixation.

Figure 5 and 6 illustrate the marked changes in ultrastructure observed. There are large numbers of "lacunae" irregularly distributed throughout the fields but

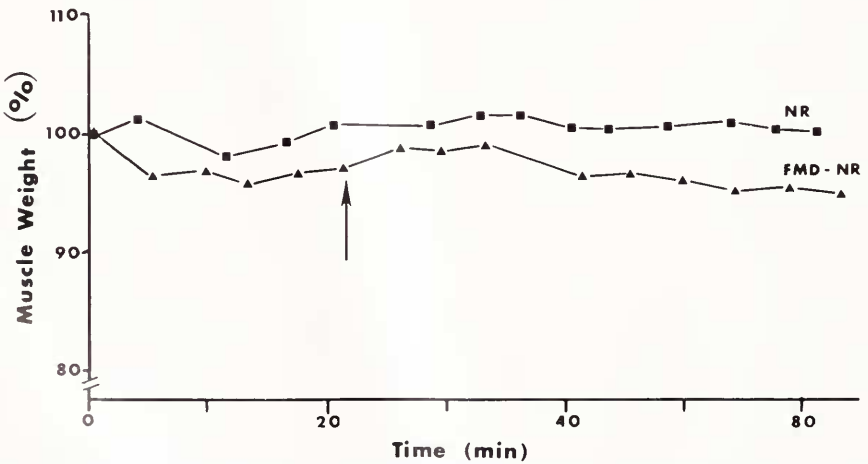

FIGURE 1. The water content of guinea pig ileum strips does not change appreciably upon exposure to 2 *M* FMD and after transfer to normal Ringer's (NR). Arrow indicates the change from FMD or NR. Top curve is the control muscle maintained in NR throughout the experiment. See Materials and Methods for experimental procedure.

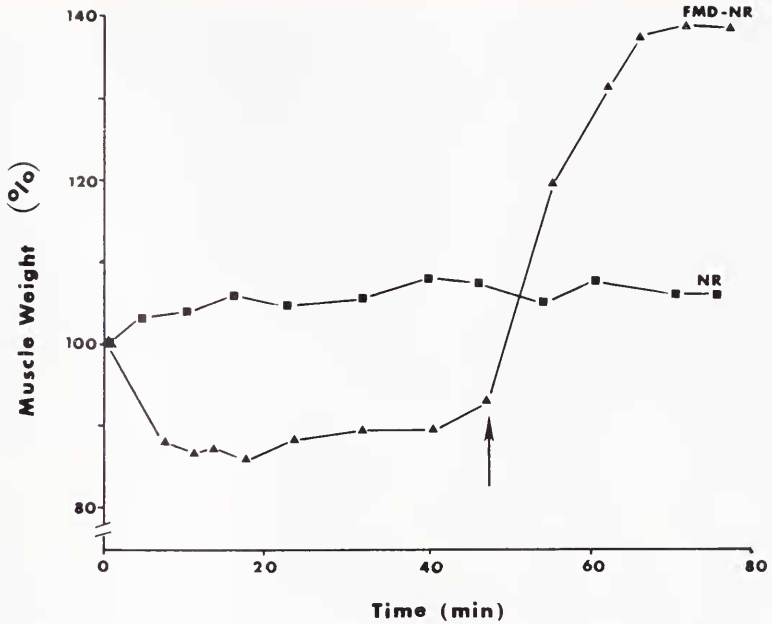

FIGURE 2. Frog sartorius muscles equilibrated in 2 *M* FMD increase markedly their water content when brought back to normal Ringer's (arrow). Muscle marked NR was maintained in normal Ringer's throughout the experiment. Experimental procedure is explained in Materials and Methods.

always lying at the level of the Z bands of the myofibrils. In these regions, the T-tubules are swollen but maintain their close association with the membrane of the terminal cisternae of the SR.

Recovery of contractility in FMD-blocked muscles transferred to isosmotic EG. To test whether the observed osmotic shock was *per se* the cause of the irreversibility of the uncoupling action of FMD on skeletal muscles, we performed experiments avoiding the occurrence of drastic osmotic changes. Pairs of sartorius muscles were exposed to a 1.0 *M* FMD solution (1,190 mOsm) for a period sufficiently long to produce a complete loss of contractility at this lower FMD concentration. These muscles were then transferred to a 1 *M* EG solution (1,210 mOsm). Ethylene glycol has been employed in similar concentrations to produce E-C uncoupling of frog muscles by an osmotic shock (Sevcick and Narahashi, 1972), but, by itself and in contrast to what we have shown with FMD, it does not impair muscle contraction (Caputo, 1968).

Both the water content of the muscles and their contractility in response to direct electrical stimulation were determined in these preparations. Figure 7 shows that the water content of these muscles decreases slightly after transfer to the EG solution, indicating that this alcohol does not penetrate freely into the muscle fibers. Figure 8 illustrates the time course of the blockade of a muscle exposed to 1 *M* FMD and the partial recovery of contractility after 24 min in EG. As emphasized in the discussion, the reduced force of contraction of muscle treated by FMD may be due to the occlusion of a fraction of the T-tubules.

Structure of muscles fixed after successive exposure to FMD and EG. Figure 9 shows a survey field of a sartorius muscle fiber exposed sequentially to 1.0 *M* FMD and isosmotic solution prior to fixation with a glutaraldehyde solution that also contained 1.0 *M* EG. The appearance of the relaxed myofibrils and the associated

FIGURE 3. Frog sartorius muscle fixed in glutaraldehyde in presence of 2 *M* formamide (see Materials and Methods). The myofibrils (in this instance contracted) and associated SR and T-system membranes are essentially normal in appearance. Arrows indicate triads situated, as is characteristic of this muscle, at the Z band level. Scale bar: 1 μm.

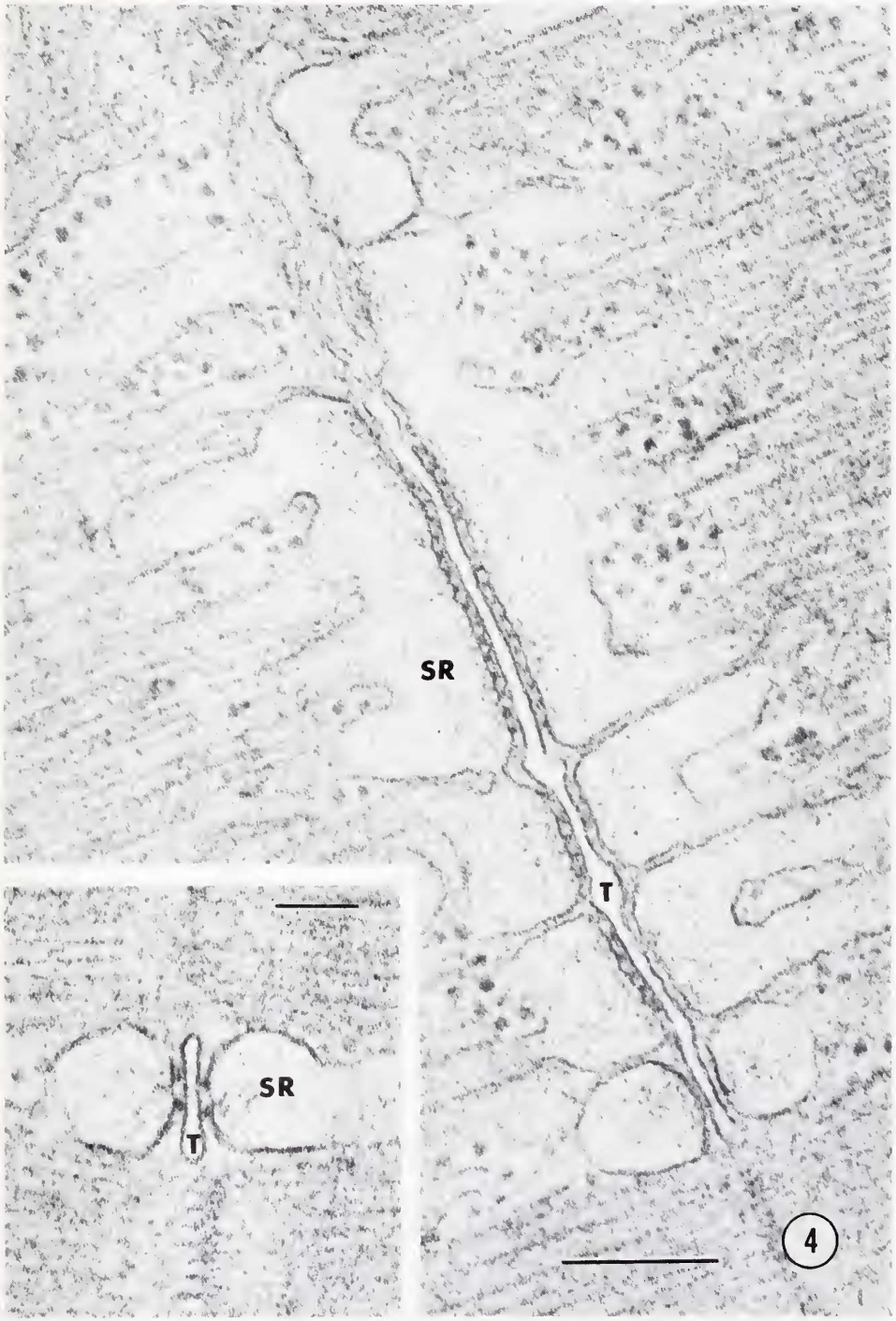

FIGURE 4. Material prepared as in Figure 3, illustrating details of the triad configurations. This field includes an extensive T-tubule profile (T), flanked by terminal cisternae (SR). The width of the triad gap and the spacing of the foot or pillar processes stemming from the SR are as in conventionally fixed material. The components of the triad are further illustrated in the inset, in which the T-tubule is transversely sectioned: note the normal disposition of the foot processes. Scale bars: 0.25 μm; inset, 0.1 μm.

FIGURE. 5. Frog sartorius muscle sequentially soaked in 2 *M* formamide, washed in Ringer's and fixed in glutaraldehyde. (See Materials and Methods for times of treatment). In this survey field, the obvious abnormality is the presence of 'lacunae' (arrows), irregularly scattered through the material, of varying size but invariably lying at the Z band levels. These are further illustrated in Figure 6. Scale bar: 1 μm.

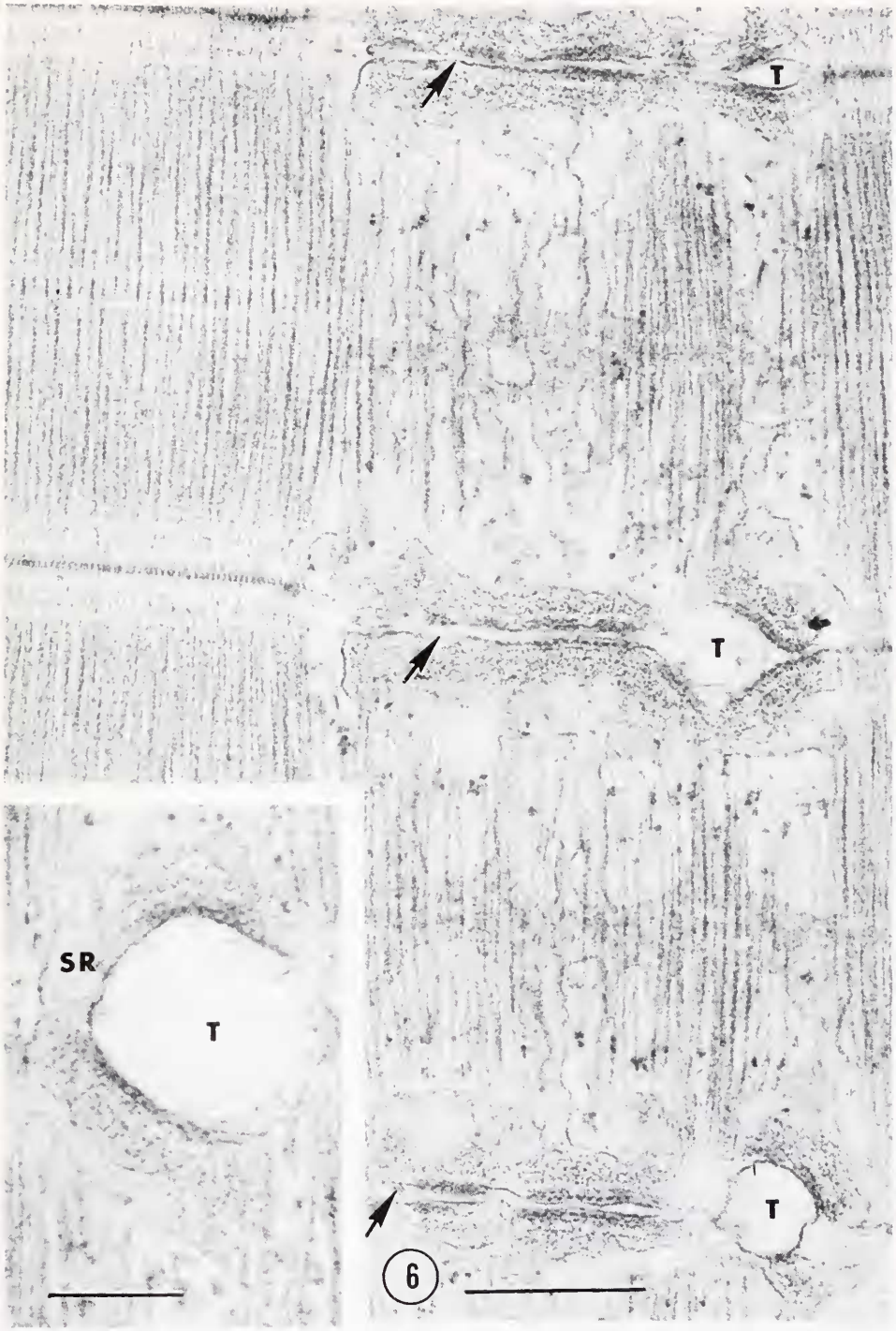

FIGURE 6. Material fixed as in Figure 5, at higher magnification. Portions of three triads are included in tangential section in this field, encircling the underlying myofibril. These are 'normal' in appearance for part of their course (arrows) but on the right of the field (T) the medial T-tubules are swollen to varying degrees, providing the Z-level 'lacunae' seen in Figure 5. As in this instance, the swelling is often irregular along the course of an individual tubule, occurring primarily between or at the periphery of the circumfibrillar triads. However, as shown in the inset, the swelling sometimes affects the triad itself. This micrograph includes a severely abnormal triad: the T-tubule is grossly swollen, but retains its original association with the terminal cisternae (SR). Scale bars: 0.5 μm; inset, 0.25 μm.

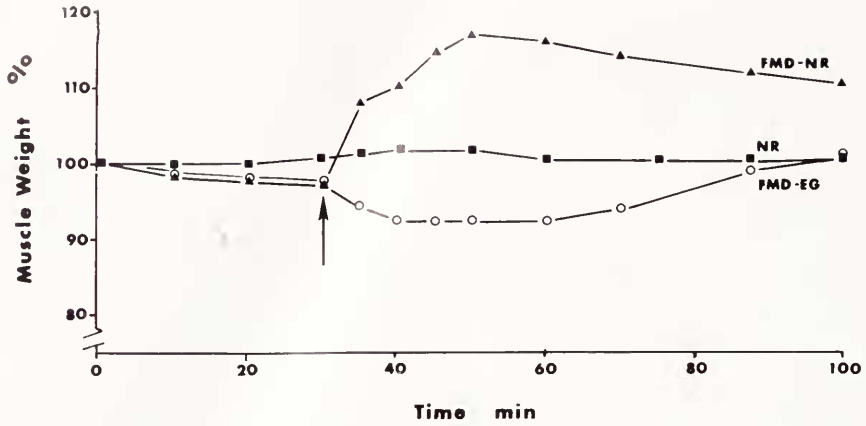

FIGURE 7. Changes in the water content of two frog sartorius muscles equilibrated with 1.0 *M* FMD and transferred to either normal Ringer's (FMD-NR) or 1.0 *M* EG (FMD-EG). The curve marked NR was obtained with a muscle maintained in normal Ringer's throughout the experiment. Arrow indicates the moment in which the muscles were changed from the FMD solution. See Materials and Methods for an explanation of the experimental procedure.

triadic junctions is essentially normal. In particular the tubular swellings obvious in Figures 5 and 6 are absent.

DISCUSSION

The results of the experiments described above demonstrate the occurrence of an osmotic shock in frog sartorius muscles transferred from FMD solutions to normal isotonic Ringer's. The fact that in any given field of the electron micrographs obtained from these muscles not all the T-tubules are swollen suggests that only some of these links with the plasma membrane remain open under these conditions. Quantitative analysis of this effect is rendered very difficult by the irregularity of the swelling, which occurs primarily between the fibrils or at the edge of the circumfibrillar triads.

The open tubules may be those that become swollen by the inflowing isotonic fluid. The non-swollen tubules, which have an essentially normal appearance, are possibly those that have sealed off during the FMD treatment becoming, effectively,

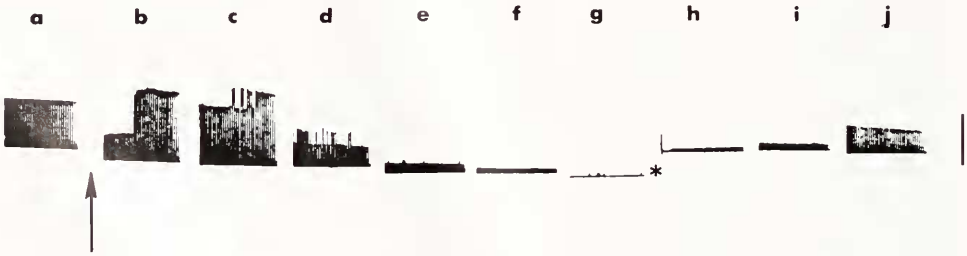

FIGURE 8. Time course of the blockade of contractility of a frog sartorius muscle immersed in 1.0 *M* FMD and its partial recovery upon transfer to 1.0 *M* EG (*). *a* records the control twitch tension developed in response to direct electrical stimuli at a frequency of 1 Hz. At arrow, FMD was added to the bath solution. *c*, *d*, *e* and *f* were recorded 3, 6, 12, and 24 min after. *f* illustrates the blockade after 32 min in FMD. *h*, *i* and *j* were recorded after placing the muscle in EG for 6, 12, and 24 min. Vertical calibration, 0.5 g; recording times, 1 min.

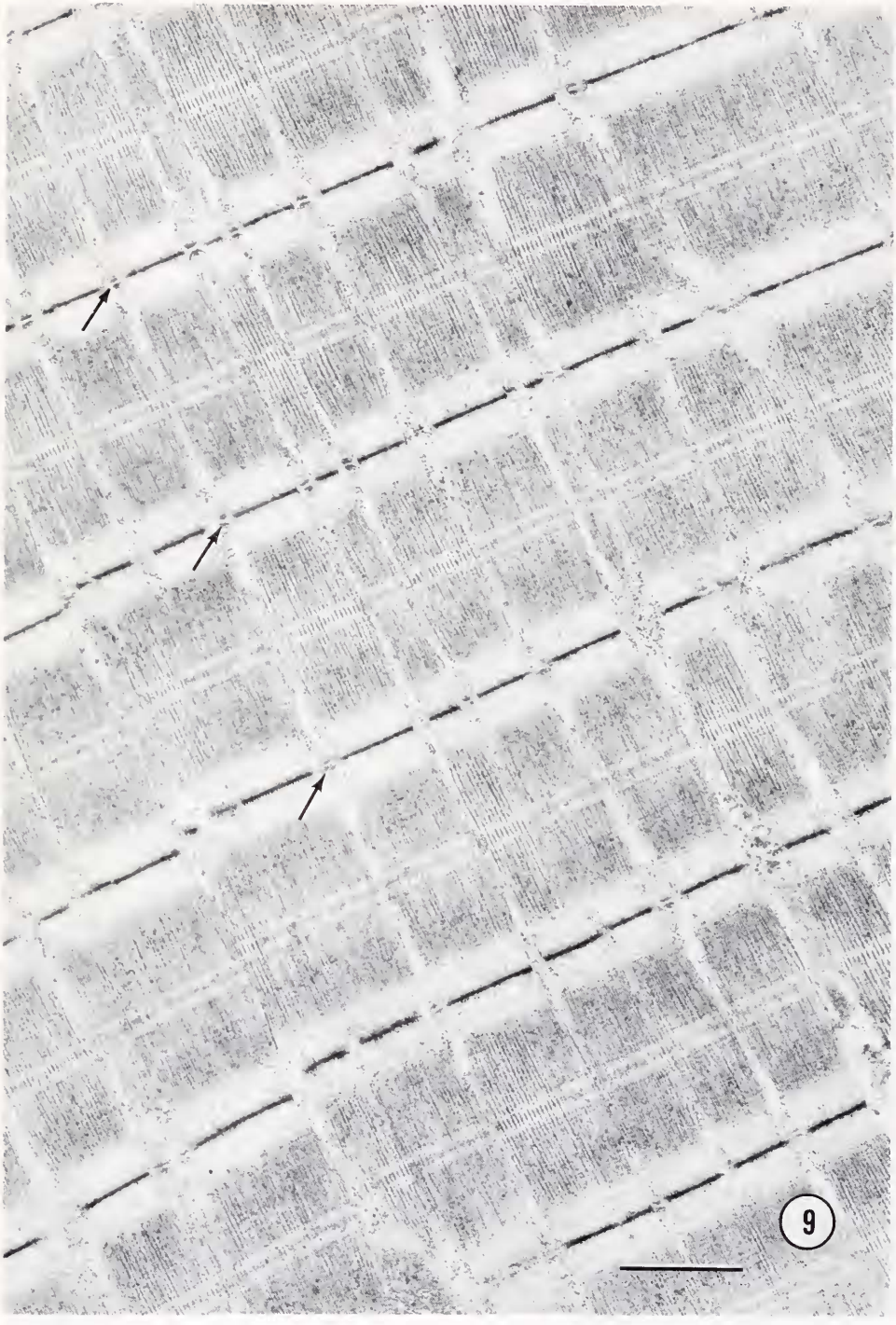

FIGURE 9. A survey field of sartorius muscle soaked sequentially in 1 *M* formamide and isosmotic EG prior to fixation in glutaraldehyde. The appearance of the myofibrils (in this instance, relaxed) and associated triad junctions (arrows) is essentially normal; in particular, the T-tubule swelling seen in Figures 5 and 6 is absent. Scale bar: 1 μm.

intracellular structures. In this respect, they would be similar to the terminal cisternae of the sarcoplasmic reticulum, which do not appear to be noticeably altered or swollen in any of the micrographs. Conversely, it is possible that swelling occurs only in sealed tubules, but this does not affect the functional interpretation.

Thus, the irreversibility of the uncoupling action of FMD on frog skeletal muscle transferred to normal Ringer's may be attributed both to the sealing of many of the T-tubules and to the swelling, and consequent loss of function, of others. In both instances, the normal triadic structure and/or function would be altered, resulting in complete loss of E-C coupling.

This conclusion is supported by the two above-mentioned observations: 1) In guinea pig ileum, a smooth muscle that lacks a tubular system, there is no obvious osmotic change when FMD-equilibrated strips are placed back in isotonic Krebs-Ringer's where the block of contractility is completely reversed. 2) When an osmotic shock is avoided, by transferring frog sartorius muscles to isosmotic EG solutions, there is a slow and partial recuperation of contractility suggesting that enough of the T-tubules remain open to permit effective E-C coupling.

We have observed changes in the after-potentials of spikes induced in muscles transferred to normal Ringer's after FMD-blockade which further suggest the occurrence of tubular disruption (Escalona de Motta *et al.*, 1982). This led us to propose that FMD exerts two separate effects on muscle contractility: a) a direct reversible inhibition, similar to that observed in guinea pig ileum, probably related to an interference with the activating action of Ca^{2+} on the contractile machinery; and b) an irreversible effect occurring only when skeletal muscles equilibrated in hypertonic FMD solutions are suddenly brought back to normal saline.

The present observations emphasize that FMD has a direct inhibitory action on the E-C coupling process that reverses slowly when the amide is removed from the preparation, avoiding drastic osmotic changes. FMD must then be included among the permeant solutes suitable for uncoupling excitation from contraction. However, as reported earlier (Escalona de Motta *et al.*, 1982), compared to other agents in this category, FMD treatment is far more gentle and better preserves the electrical parameters of the muscle fibers.

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